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ON THE TRYPSIN SENSITIVITY OF GAMETE CONTACT AT FERTILIZATION AS STUDIED WITH LIVING GAMETES IN CHLAMYDOMONAS[1]

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Specific sensitivity to enzymes has been a useful means for the characterization of sex cell interaction at fertilization by revealing the nature of the participating functional structures of the gamete surfaces (*cf.* Metz, 1954; *cf.* Runnström, Hagström and Perlmann, 1959; *cf.* Metz, 1967; Brock, 1965; Crandall and Brock, 1968; Taylor and Orton, 1967). Appropriate enzymes may be expected to interfere with one or more steps in the attachment and fusion processes at fertilization or to reduce its specificity. Such effects have been described in sea urchin fertilization. Trypsin treatment reduces the fertilizability of the eggs, probably by elimination of the sperm receptor substance(s) (Tyler and Metz, 1955; Runnström and Kriszat, 1960). Trypsin treatment of the eggs facilitates cross-fertilization in the sea urchin (Hultin, 1948ab; Tyler and Metz, 1955; Hagström, 1959) and eliminates self sterility in the hermaphroditic ascidian *Ciona* (Morgan, 1939; Bohus-Jensen, 1958). These effects of trypsin indicate that the components of the interacting systems at fertilization are proteinaceous or are closely associated (*i.e.*, anchored to) proteinaceous material. In addition, proteases provided the first evidence for the protein nature of isolated attachment substances (Tyler and Fox, 1940).

The isogametic copulation of the Chlorophycean, *Chlamydomonas,* provides a simple model-like example of sexual differentiation and of fertilization events for analysis. In the isogamous, heterothallic species used in these studies, gamete union proceeds in two steps (*cf.* Coleman, 1962; Wiese and Jones, 1963). The initial mating type reaction effects a specific agglutinative adhesion between gametes of opposite sex. This adhesion occurs at the flagella tips and implies the interaction of specific mating type substances (*cf.* Wiese, 1965). Subsequent to the mating type reaction the agglutinated gametes unite into pairs: In *C. moewusii* and *C. eugametos* two sexually different gametes establish papillar contact at the basis of their flagella. The papillae fuse and form a protoplasmic bridge which connects the two cells resulting in a peculiar prezygotic stage, the *vis-à-vis* pair. After papillar attachment the agglutinated flagella separate, and the *vis-à-vis* pair moves and behaves as one physiological unit. Finally, after 18–36 hours, the two gametes fuse completely and caryogamy occurs. The mating type substances have been isolated and act as isoagglutinins (*cf.* Wiese, 1965): each component makes gametes of its respectively opposite sex agglutinate one with another.

These isoagglutinations are assumed to result from bi- or multivalency of the isoagglutinins, in keeping with immunological doctrine. Trypsin and pronase have

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been used to examine the functional structure and chemical composition of the isolated mating type substances (Wiese and Wiese, in preparation). This paper deals with the effects of trypsin on the mating type substances *in situ* and on the entire copulation process. Trypsin was selected because its pH-optimum coincides with that for mating in *Chlamydomonas* (Wiese, unpublished) and because the mode of its proteolytic action is known (Bergmann and Fruton, 1941).

Material and Methods

The experiments were performed with *Chlamydomonas eugametos* and with *C. moewusii* syngen I which has been identified by complete sexual compatibility with the Indiana strains Collection No. 96/97 (*cf.* Starr, 1964). The species specificity of the mating type reaction during the trypsin treatment was checked with gametes of *C. reinhardti, C. mexicana*, and *C. moewusii* syngen II (Indiana Collection No. 792/793, sexually incompatible with No. 96/97 and with *C. eugametos*). These two strains were earlier designated as *Chlamydomonas* spec. (cf. Wiese, 1965). We label No. 793 as the (−) strain and No. 792 as the (+) strain since the latter's gametes are responsible for the locomotion of the *vis-à-vis* pair. By this feature strain No. 792 corresponds to the (+) sex of *C. moewusii* syngen I (*cf.* Lewin, 1952) and to the male sex of *C. eugametos* (*cf.* Wiese, 1965).

All strains including *C. reinhardti* were cultured on KNOP-agar (Wiese, 1965) at 19 ± 1° C and with an illumination of 1200 Lux given in a light cycle of 16^h light and 8^h dark. [*C. reinhardti* grows excellently on nitrate as sole nitrogen source.]

The vegetative cells were induced to undergo gametogenesis by flooding the agar slants overnight with sterile 0.02 *M* TRIS-buffer, pH 7.6. In the morning the gametes were washed by centrifugation at 180 g for 5 minutes and resuspension in the TRIS-buffer containing 0.01 g/l $CaCl_2$, 0.01 g/l K_2HPO_4, and the respective $MgSO_4$-concentration applied, *i.e.*, 0.025 or 0.0125%. The cell density of the suspensions, as determined by hematocrits, was always adjusted for 4 cubic millimeters of packed cells per milliliter.

Salt free, 2 x crystallized trypsin (Worthington Biochemical Corp., Freehold, New Jersey) and 5 x crystallized soybean inhibitor (Nutritional Biochemical Corp., Cleveland, Ohio) were used in the experiments.

With respect to the mating type reaction, the degree of the sexual reactivity of treated and control gametes was assessed microscopically and scaled from − (no agglutination) to +++ (agglutination of virtually all gametes). Since the mating type reaction is an obligatory prerequisite to pairing, the intensity of the mating type reaction can also be determined quantitatively by the ratio of paired to unpaired cells provided that the trypsin influence upon pairing, as described in this paper, is eliminated by application of trypsin-inhibitor. On the other hand, when the mating type reaction is not affected the number of pairs obtained in experimental and control mixtures provides a selective measure of the inhibition of pair formation. Additional details are given in the text.

Results

Trypsin exerts a distinct inhibitory action on fertilization of *Chlamydomonas moewusii* and *C. eugametos*. By varying trypsin concentrations and manner of

application it was possible to show that both steps in fertilization, namely the mating type reaction and the pairing, are trypsin-sensitive. Both events can be blocked selectively. The "vegetative" functions of the *Chlamydomonas*-gametes including those prerequisites for copulation such as flagellation and motility, are not visibly affected by the trypsin treatments employed. Gametes show undiminished motility even after 24 hours at 26° C in 0.1% trypsin, the strongest solution used.

The trypsin-sensitivity of the pairing

A selective action of trypsin on this second phase of the copulation process can only be demonstrated if the trypsin treatment leaves the initial agglutinative mating

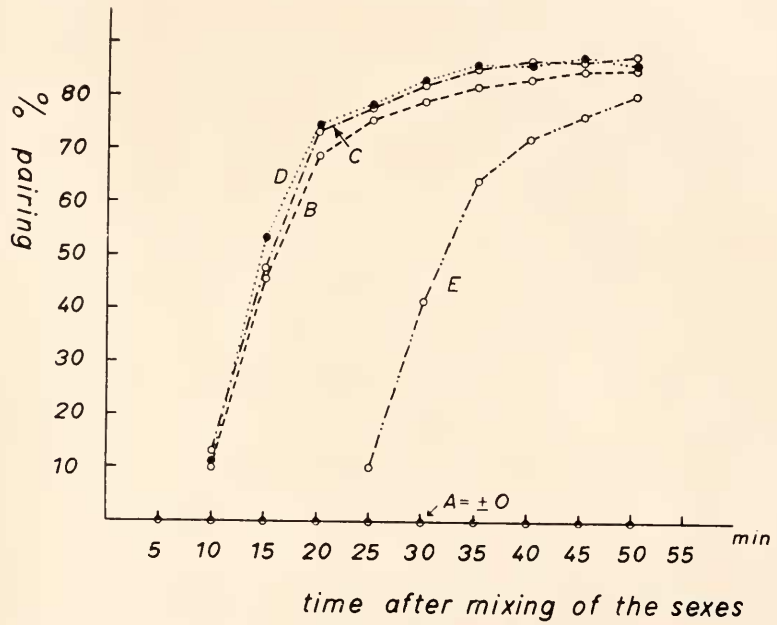

FIGURE 1. Trypsin inhibition of the pairing in *C. moewusii* (for detailed information see text). A. Complete inhibition by 0.025% trypsin. B. Trypsin effect entirely counteracted by 0.05% trypsin-inhibitor. C. Pair formation in the buffer control. D. Failure of 0.05% trypsin-inhibitor to affect pairing. E. Approximately uninhibited pairing in 0.025% trypsin after delayed application of 0.05% trypsin-inhibitor.

type reaction unaffected. In preliminary experiments pairing was found to be markedly reduced in 0.025% trypsin solutions, whereas the capacity of the flagella tip to agglutinate was not affected by this trypsin concentration. Gynogametes and androgametes which have been pretreated with this concentration for two hours agglutinate immediately and quantitatively upon mixing. Therefore, 0.025% trypsin was used to investigate the trypsin sensitivity of the pairing.

The selective action of this trypsin concentration upon pairing in *C. moewusii* syngen 1 is shown in Figure 1. Gamete suspensions of both sexes were prepared overnight as outlined in the methods sections. In the morning the two gamete

types were mixed and immediately 50 ml of the mixed suspension were added to each of the following samples:

A. 50 ml Tris-buffer + 25 mg trypsin + 6.25 mg $MgSO_4$
B. 50 ml Tris-buffer + 25 mg trypsin + 6.25 mg $MgSO_4$ + 50 mg trypsin inhibitor
C. 50 ml Tris-buffer + 6.25 mg $MgSO_4$
D. 50 ml Tris-buffer + 6.25 mg $MgSO_4$ + 50 mg trypsin inhibitor
E. 50 ml Tris-buffer + 25 mg trypsin + 6.25 mg $MgSO_4$. 50 mg trypsin inhibitor (dissolved in additional 10 ml buffer) were added to this sample after 15 min.

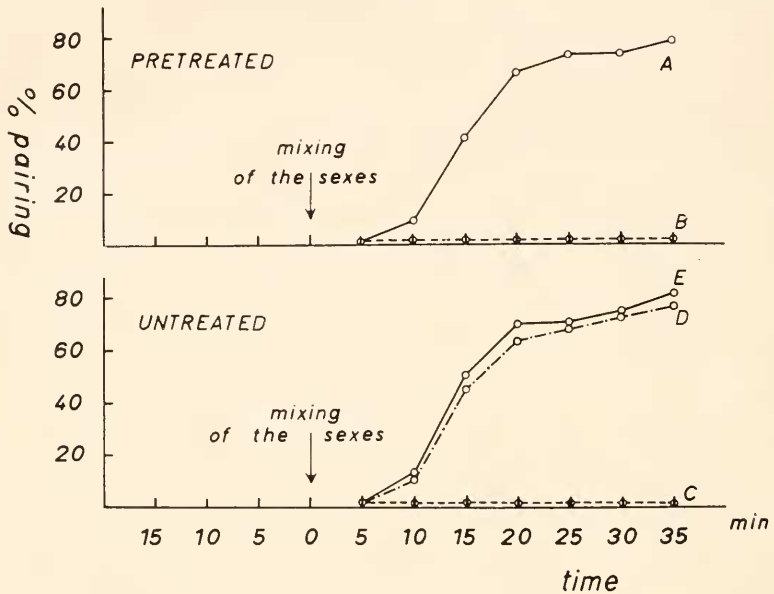

FIGURE 2. Trypsin inhibition of pair formation in *C. moewusii*. Different application of trypsin (at mixing of the sexes or as pre-incubation) in presence or absence or trypsin inhibitor. A and B: Gametes pretreated with 0.025% trypsin 15 min. before mixing of the sexes. A. Trypsin inhibitor (0.05%) added at mixing; trypsin content adjusted to 0.025% final concentration. B. Without inhibitor. C, D and E: Same material not pretreated. Added at mixing of the sexes were C. 0.025% trypsin, D. 0.025% trypsin and 0.05% inhibitor, and E. 0.05% inhibitor.

Microscopic examination of the samples showed that during the entire course of the experiment the trypsin-treated gametes were not impaired with respect to flagellation, motility, and agglutinability. However, the trypsin-treated samples showed markedly reduced pair formation. In a representative experiment (Fig. 1) the control gamete mixture yielded over 80% pairing (curve C 87.2%, curve D 86.3%) whereas the trypsin-treated sample (curve A) was practically devoid of pairs. Trypsin-inhibitor, added simultaneously with trypsin at the mixing of the gametes, yielded full pairing (curve B, 85.6%). Addition of trypsin-inhibitor 15 minutes after trypsin application still yielded 80.6% pairing (curve E).

The data of Figure 1 clearly show that treatment with trypsin in the appropriate concentration selectively inhibits pair formation. This effect is assumed to result from the proteolytic action of the enzyme since the effect is not obtained in the presence of trypsin-inhibitor.

The failure to pair could be due to an enzymatic interference with the process of pair formation or to the enzymatic elimination of functional components at the prospective attachment sites. Such functional components essential for papillar attachment and adhesion could exist either before the mating type reaction, or could be formed or set into action only after the mating type reaction has ensued.

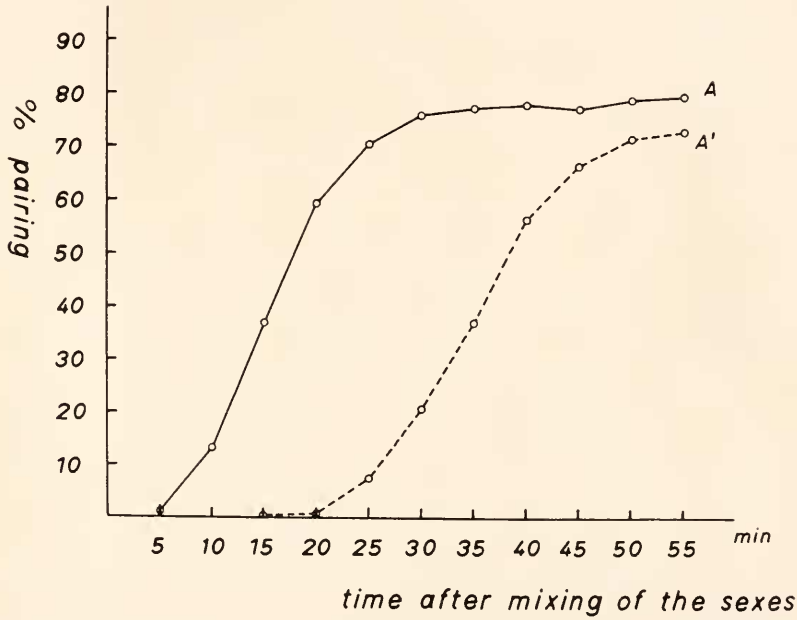

FIGURE 3. Trypsin-sensitive phase of pair formation in *C. mocvusii*. After mixing of both gamete types samples were taken at 5 min intervals, incubated with 0.025% trypsin for 15 min and fixed afterwards (A'-data). Compared with the normal formation rate of pairs (Curve A) each A' value has to be related to the percentage of pairs originally present in the sample in question as well as to the percentage to which the pairing has meanwhile proceeded.

According to Figure 1 (curves A and C) trypsin prevents any pairing when added simultaneously with the mixing of the two gamete types. About 5–7 min elapse between the initial agglutination and the appearance of free-swimming and fixation-resistent pairs. If pairing is prevented by the enzymatic elimination of preexisting attachment sites, the 5–7 minute time lapse would have to be sufficient to eliminate the sites. However, gametes which were pretreated with trypsin for twice that length of time, did not show any inhibition of the pairing if sufficient trypsin-inhibitor was added at the time of mixing of the sexes (Fig. 2). Evidently, inhibition of the pairing by trypsin does not result from the enzymatic destruction

of preexisting components at the gametes' apices. This fact is also indicated by the appearance of pairs after delayed addition of trypsin inhibitor (Fig. 1, E). The inhibitory effect is exerted during the formation of the plasmatic bridge.

To further explore this trypsin action, the effect of short duration treatments throughout the copulation process was investigated. A (+) and a (−) gamete suspension were mixed. At 5 minutes intervals two samples of one ml volume were taken from the mixture. One sample (A-series) was diluted with one ml

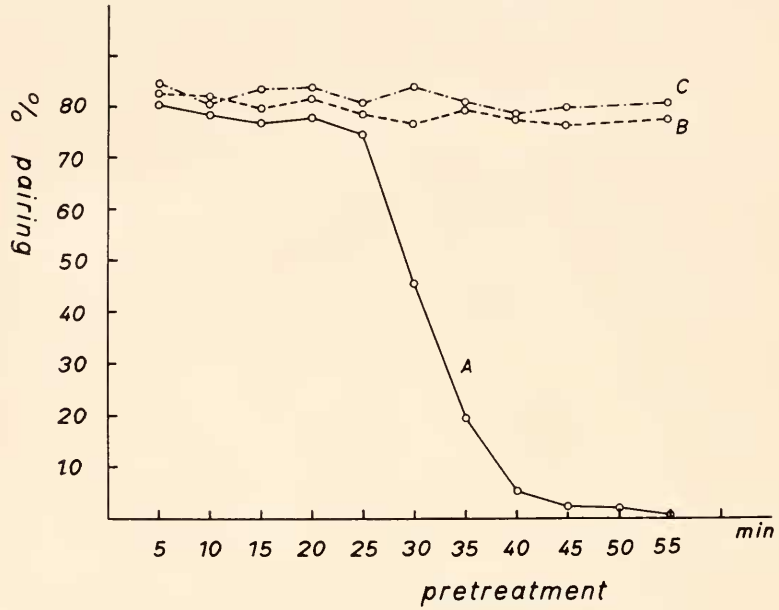

FIGURE 4. Trypsin sensitivity of the mating type reaction in *C. moewusii* as determined by the pair test. A. Inactivation of the (−) gametes. 150 ml (−) gamete suspension (8 mm³ packed cells/ml) were incubated with 0.1% trypsin. At 5 minute intervals, 5 ml samples were taken and the incubation was interrupted by adding 5 ml 0.1% trypsin-inhibitor. After addition of 10 ml untreated (+) gametes (4 mm³ packed cells/ml), the number of pairs was stated fixing each sample after 30 minutes. B. Trypsin effect upon the (+) gametes checked correspondingly with untreated (−) gametes. C. Control indicating the virtually unaltered agglutinating capacity of both gamete suspensions during the time of the experiment. 5 ml of both untreated gamete types (8 mm³ packed cells/ml) were combined in 5 minute intervals and 10 ml 0.05% trypsin-inhibitor solution were added. The percentage of pairs formed in each sample within 30 minutes is determined.

TRIS-buffer containing 0.0125% $MgSO_4$ and immediately fixed by addition of 2 drops of aqueous Lugol's solution. To the other sample one ml of the buffer containing 0.05% trypsin and 0.0125% $MgSO_4$ was added and this sample (A′ series) was fixed after 15 minutes. Such "pulse treatment" with trypsin reveals when the trypsin-sensitive phase begins, and detects its temporal relationship to the final fusion of the gametes (Fig. 3). From the curves A and A′ it is evident that (1) trypsin stopped the pairing; (2) trypsin is able to split to a considerable degree

pairs which had been so well established that they resist fixation (in the corresponding A-sample); and (3) the ability to split pairs declines with the length of time the pairs have been established until finally the papilla fusion proceeds to a condition no longer divisible by trypsin. The special trypsin-sensitive period exists only at the beginning of the pairing stage.

In all these points gametes of *C. eugametos* gave similar results when subjected to the same experimental procedures.

The trypsin-sensitivity of the mating type reaction

The experiments reported in the previous section were conducted on the premise that trypsin, in the concentrations and exposure times used, had little if any effect on the initial mating type reaction. At higher trypsin concentrations, however, the flagellar agglutination, too, was affected in a specific manner. This particular effect was best demonstrated with a concentration of 0.1% trypsin which did not interfere with the flagellation or the locomotion of the gametes. The Mg^{++}-concentration was kept at 0.025% in order to prevent any ionic disturbance of the agglutination process. This trypsin action, too, can be prevented by addition of trypsin-inhibitor.

In a mixture of androgametes and gynogametes incubated with 0.1% trypsin at 26° C for a longer period, the initial intensive agglutination of the gametes gradually decreases and is finally, after 45–60 minutes, lost entirely. No pairs are formed. Separate pre-incubation of the two gamete types and mutual checking with untreated test gametes revealed for *C. moewusii* syngen I that it is exclusively the (−) sex which is sensitive (Fig. 4). Thus the trypsin pretreated (+) or androgametes agglutinated strongly with test (−) gametes whereas trypsin pretreated gynogametes failed to agglutinate with (+) test gametes.

The different effects exerted on the mating type reaction and on pairing permitted the use of the pair test as a quantitative measurement for the inhibition of the mating type reaction, provided that the action of trypsin on pairing was neutralized by trypsin-inhibitor added at the time of mixing of the gametes (Fig. 4).

Again, *C. eugametos* reacts in an entirely similar manner; one mating type is sensitive and the other is resistent to trypsin. The trypsin-sensitive type (female) corresponds to the (−) sex of *C. moewusii* syngen I.

Since the species specificity of copulation is associated with the mating type reaction (*cf.* Wiese, 1965), the conservation of this specificity was examined during the action of 0.1% trypsin. At 5 minutes intervals, samples of the 4 treated gamete types (*C. moewusii* syngen I (+) and (−), *C. eugametos* male and female) were combined with highly active gametes of *C. reinhardti*, *C. moewusii* syngen II, and *C. mexicana*. Any possible loss of specificity was tested by checking for the appearance of non-specific flagellar agglutination. Each sex of *C. eugametos* and *C. moewusii* syngen I was checked against both sexes of the incompatible forms; no case of a non-specific agglutination was observed.

Discussion

Among the sequence of events proceeding at copulation, the two trypsin-sensitive steps namely the mating reaction and papillar union, are well defined and can be

selectively influenced. Thus, a higher trypsin concentration and longer incubation time is required to inhibit the mating reaction than to inhibit pairing. Among the species that have been cross tested, the species specificity of copulation remains unaltered by treatment with trypsin.

The trypsin influence on the mating type reaction

The action of trypsin on the mating type reaction is characterized by its unilateral inhibition of the (−) sex alone. Likewise, in the sea urchin, the trypsin sensitivity of fertilization is unilateral and restricted to the egg whereas the fertilizing capacity of the sperm remains unaffected (Tyler and Metz, 1955; Hagström, 1959).

In *Chlamydomonas,* the unilateral or differential effect of trypsin can be explained in at least two different ways as follows: (1) the (−) mating substance but not the (+) substance is inactivated by trypsin. (2) Both the (+) and the (−) substances are inactivated by trypsin but the (+) substance is replenished continuously by a gametogenic metabolism specific to the (+) or androgamete (Förster and Wiese, 1954; Förster, 1957, 1959; Stifter, 1959; Hartmann, 1962). On the other hand the essential amount of (−) substance would be produced at gametogenesis by the gynogamete, and once destroyed by trypsin, no new (−) substance would be produced to the extent that the mating reactivity will be restored.

The fact that both isoagglutinins are sensitive to trypsin (Förster, Wiese and Braunitzer, 1956; Wiese, 1961; Wiese and Wiese, in preparation) does not decide in favour of the second alternative since the detached components (isoagglutinins) might well be more sensitive to tryptic attack than the same components *in situ.* Moreover, the capacity to produce isoagglutination requires a functional bi- or multivalent structure, whereas gamete adhesion presumably could result from interaction of univalent complementary cell surface substances. Accordingly, loss of the multivalent structure would destroy the agglutinating action of the substance in solution, but not necessarily the adhesive properties of the material at the cell surface.

Analyses of the effect of trypsin on the isoagglutinins and of the supernatants of trypsin-treated gametes are expected to answer this question. Evidence so far available indicates that a macromolecular component which is split off during the tryptic inactivation of the gynogametes, has no capacity to induce isoagglutination of the androgametes (Wiese and Wiese, in preparation). In the yeast, *Hansenula,* trypsin inactivates one mating type by splitting off its mating substance (Crandall and Brock, 1968). The mating substance of the other mating type can be detached by a snail enzyme preparation (Taylor, 1964) or by subtilisin (Taylor and Orton, 1967). In both mating types, the enzymatically detached components have here retained their capacity to combine with the complementary gametes.

A further possibility that the disappearance of the sexual activity on addition of trypsin is not a specific enzyme effect at all, but rather a differentiation back to the vegetative stage caused by the supply of a metabolizable N-component, is excluded by the non-appearance of the trypsin effect on addition of trypsin plus trypsin inhibitor. This possibility also seems highly improbable because addition of peptones, glycine, alanine, trypsin-inhibitor, and normal rabbit serum in no way

interferes with the sexual activity (Wiese, unpublished). Such an interference complicated the corresponding analysis of the conjugation process in *Paramecium* (Metz and Butterfield, 1951). Trypsin incubation of dead gamonts which were killed without destroying their mating type activity, revealed a bilateral trypsin-sensitivity of the mating type reaction (*cf.* Metz, 1954). A difference between the mating types was demonstrated by means of nitrous acid and certain formalin concentrations. These inactivated the reactivity in one mating type only and offered a hint to the nature of the reactive groups involved (*cf.* Metz, 1954).

The influence of trypsin on pair formation

The specific elimination of pairing in an organism with a two-step gamete adhesion and fusion sequence was first demonstrated in *Chaetomorpha* after application of subtilisin (Köhler, 1956). It was concluded that the different enzyme sensitivities of pair formation and flagella agglutination, respectively, resulted from different chemical bases of the two steps. In *Chlamydomonas,* the two steps do result from two different mechanisms as shown by their different sensitivity to SH-reagents and to laurylsulfate (Wiese and Jones, 1963). This difference, however, cannot be derived directly from the different sensitivity to trypsin, since there are apparently no prospective attachment sites at the papillae comparable to the flagella agglutinin. Trypsin-pretreated gametes do pair when trypsin-inhibitor is added at the moment of mixing of the two gamete types. Addition of inhibitor after the mixing (Fig. 1, E) is as effective and even more informative in case such attachment sites would not be existent (or susceptible) before the final completion of the flagella attachment, *i.e.,* if the capacity to make papilla attachment would arise as an induction effect from the prior flagella attachment. The effect on pairing extends to an elimination of the morphogenetic process of the bridge formation in concentrations of 0.025% trypsin and higher. Slightly lower concentrations may permit bridge formation to proceed but render the established pairs so weakened that the pair mates separate on subsequent fixation. The trypsin-sensitive period extends beyond the stages at which the papilla attachment is achieved, the flagella attachment discontinued, and the decision made on the different behavior of the (+) and the (−) flagella. During this period, even pairs which are resistant to fixation may still be split by trypsin (Fig. 3). The mates of older pairs, however, are no longer separable.

Summary

Trypsin affects the copulation of isogametes in *Chlamydomonas* (*Chlorophyceae*) in two ways. In low concentrations it rapidly suppresses pair formation by interfering with the formation of the protoplasmic bridge between the gynogamete and the androgamete. In the early phase of its formation the bridge may even be split by the action of trypsin. In higher concentrations, trypsin additionally affects the mating type reaction by destroying the capacity of the gynogametes to agglutinate with the androgametes. The agglutinating capacity of the androgametes is not impaired by trypsin. Trypsin does not affect the species specificity of the attachment mechanism within the system tested.

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